Verfahren und Medikament zur Hemmung der Expression eines vorgegebenen Gens

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Abstract of **DE19956568**

The invention relates to a medicament containing at least one double-stranded oligoribonucleotide (dsRNA) designed to inhibit the expression of a target gene. According to the invention, one strand of the dsRNA is at least in part complementary to the target gene.

Treatment and medicine for the inhibition of the expression of a given gene.

The invention concerns a treatment according to the genus of claim 1. It concerns, moreover, a medication and the usage of a double stranded oligoribonucleotide.

Such a treatment is known from the published WO 99/32619. The familiar treatment targets the inhibition of the expression of genes in cells of invertebrates. For this it is necessary that the double stranded oligoribonucleotide shows an identical sequence with a length of at least 50 bases, to that of the target gene. To attain an efficient inhibition a length of the identical sequence of 300 to 1000 base pairs are necessary. The cost of producing such an oligoribonucleotide is very high.

The DE 196 21 919 C2 describes an anti-sense-RNA with special secondary structures, whereby the anti-sense-RNA is present in the form of a coding vector. With the anti-sense-RNA one is dealing with a RNA molecule that is complementary to areas of the mRNA. By connecting to these areas the inhibition of the gene expression is brought into effect. This inhibition can, in particular, be used for diagnosis and/or therapy of sicknesses, e.g. tumor sicknesses or viral infections. The anti-sense-RNA unfortunately must be delivered to the cells in large quantities, which are at least as large as the amount of mRNA. The effectiveness of the familiar anti-sense-treatment is not particularly high.

A medication is known from US 5,712,257 that contains incorrectly paired double stranded RNA (dsRNA) and biologically active incorrectly paired pieces of dsRNA in the form of a ternary complex with a surface active agent. The dsRNA used consists of a synthetically produced nucleic acid single strands without a defined vase sequence. The single strands are not in regular so-called "Not-Watson-Crick" base pairings with each other so that incorrectly paired double strands are formed. The known dsRNA is used for the inhibition of the propagation of retroviruses, such as HIV. The propagation of the virus can be inhibited if dsRNA that is not sequence specific is delivered to the cells. This leads to an induction of interferon, through which the virus propagation is supposed to be inhibited. The inhibiting effect as well as the effectiveness of this treatment is small.

From Fire, A. et al, NATURE, Vol. 391, pp. 806 it is known that dsRNA, of which one strand has sections complementary to a gene of a filament worm that is to be inhibited, is highly effective in inhibiting that gene. The view is that, the special effectiveness of the dsRNA used in cells of the filament worm is not based on the anti-sense-principles, but instead are possibly the result of the catalytic properties of the dsRNA, or the induced enzymes. Nothing is mentioned of the effectiveness of specific dsRNA relating to the inhibition of gene expression, especially in mammal and human cells, in this article.

The task of this invention is to eliminate the disadvantages according to the status of technology. It should especially specify a treatment and medication as efficient as possible, as well as a procedure as efficient as possible to produce a medication, with which a especially effective inhibition of the expression of a given gene is possible.

This problem is solved by the characteristics of the claims 1, 2, 33, 34 and 66 and 67. Advantageous designs arise from claims 3 to 32, 35 to 65 and 68 to 100.

According to the requirement of the procedural side of the invention the area complementary to the target gene can have at the most 49 consecutive nucleotide pairs.

The oligoribonucleotides the invention is dealing with are such that they at least show a defined nucleotide sequence in sections. The defined section can be limited to the complementary region. However, it is also possible that the double stranded oligoribonucleotide show a defined nucleotide sequence as a whole.

It has surprisingly been shown, that already with a length of the complementary area of at most 49 base pairs can achieve inhibition of the expression of the target gene. Appropriate oligoribonucleotides can be supplied with a low manufacturing cost.

In particular dsRNA with a length of more than 50 nucleotide pairs induce specific cellular mechanisms in the human and mammal cells, e.g. the dsRNA dependent protein kinase or the 2-5A-system. That leads to the elimination of the interference effect caused by dsRNA with one defined sequence. This causes the protein biosynthesis in the cell to be blocked. This disadvantage is eliminated by this particular invention.

Furthermore, the uptake of dsRNA with a short chain length into the cell or the cell nucleus is easier as opposed to the long chained dsRNAs.

If the dsRNA is used as an active ingredient it has proven to be advantageous to pack the dsRNA in micellular structures, preferably in liposomes. The dsRNA can also be enclosed in natural viral capsids, capsids produced in an enzymatic way, capsids produced chemically or structures derived from them. The aforementioned characteristics make it possible for the dsDNA to infiltrate the given target cells.

According to another arrangement characteristic the dsRNA has 10 to 1000 preferably 15 to 49 base pairs. Te dsRNA can therefore be longer than the complementary region of the target gene. The complementary region can be arranged terminally or can be activated in the dsRNA. Such a dsRNA or a similarly coded vector can be manufactured synthetically or through popular enzymatic techniques.

The gene to be inhibited is expediently exprimated into eukaryotic cells. The target gene can be chosen from the following group: oncogene, cytokine gene, id-protein-gene, development gene, prion gene. It can also be exprimated into pathogen organisms, preferably plasmodium. It can be part of, preferably human pathogens, virus or viroids. The proposed medicine allows the therapy of genetically controlled diseases, such as cancer, viral sicknesses or morbus Alzheimer.

The virus or viroid can also be a animal or plant pathogen virus or viroid. In this case the drug pertaining to the invention allows the treatment of animal and plant sicknesses as well.

According to another design characteristic the dsRNA takes on a double stranded form in some sections. The complementary section is formed from two separate RNA single strands or from a self complementary sections of a, preferably circularly shaped topologically closed RNA single strand.

The ends of the dsRNA can be modified in order to counteract degradation in the cell or dissociation in the single strands. Dissociation occurs especially when using low concentrations or short chain lengths. For particularly effective inhibition of the dissociation the cohesion resulting from the nucleotide pairs in the complementary region can be increased through at least one, preferably two, further chemical catenations. The dsRNA pertaining to the invention, whose dissociation is lessened, shows a higher stability against enzymatic and chemical degradation in the cell as well as the organism.

In particular, with the use of the vector pertaining to the invention the complementary section can be formed of self complementary regions or a RNA hairpin loop. The nucleotides are chemically modified in the loop region between the double stranded structure in order to protect from degradation.

The chemical catenation is expediently formed of a covalent or ionic bond, a hydrogen bridge bond, hydrophobic interactions, preferably van der Waals or stapling interactions, or through metal ion coordination. It can also be formed according to a very advantageous arrangement characteristic on one preferably on both ends of the complementary area.

It has also proved to be advantageous for the chemical catenation to be formed by means of one or more bonding groups, where the bonding groups are preferably poly-(oxyphosphinicooxy-1,3-propandiol) and/or polyethylenglycol-chains. The chemical catenation can also be formed by purinanaloga used by purins in the complementary area. It is further of advantage, that the chemical catenation in the complementary section is formed by the azabenzol units released into the complementary area. Furthermore, it can be formed by the branched nucleotide analoga present instead of nucleotides.

It has also be shown to be expedient, that for the creation of the chemical catenation at least one of the following groups be used: methyl blue, bi-functional groups, preferably bis-(2-chlorethyl)-amine; N-acetyl-N'-(p-glyoxyl-benzoyl)-cystamine; 4-thiouracil; psoralen. Further, the chemical catenation can be formed by thiophosphoryl groups placed on the ends of the double stranded section. The chemical catenation is preferably created by triple helix bonds on the end of the double strand section.

The chemical catenation can expediently be induced by ultraviolet light.

According to another especially advantageous form it is designed so that the dsRNA is bound associated or surrounded by at least one viral envelope protein taken from a virus or synthetically manufactured.

The envelope protein can be derived from the polyomavirus. It can contain the envelope protein of the virus protein 1 (VP1) and/or the virus protein 2 (VP2) of the polyomavirus. The aforementioned characteristics make is considerably easier to introduce the dsRNA into the cell.

Preferably one side is turned towards the inside of the capsids or capsid-like structure during the formation of a capsid or capsid-like structure out of the envelope protein. The construct formed is especially stable.

The dsRNA can be complementary to the primary or to the processed RNA-transcript of the target gene. The cell can be a cell of a vertebrate or a human cell.

Futhermore, according to the requirements of the invention a drug with at least one oligoribonucleotide with a double strand structure is needed for the inhibition of the expression of a given gene, where one strand is at least partially complementary to this gene. It has surprisingly been shown that such dsRNA is suitable as a drug for the inhibition of the expression of a given gene in human cells. The inhibition already takes effect when concentrations of it are used that are at least one order of magnitude smaller than those used when using single stranded oligoribonucleotides. The drug associated with the invention is highly effective. Few side effects are anticipated.

According to further requirement of the invention a drug with at least one vector for the coding of double stranded oligoribonucleotides (dsRNA) for the inhibition of the expression of a given gene is provided for, where one strand of the dsRNA is at least partially complementary to this gene. The proposed drug shows boasts the aforementioned advantages. Through the usage of a vector manufacturing costs can be saved.

One especially advantageous design is that the complementary area has a maximum of 49 consecutive nucleotide pairs. It has surprisingly been shown that already with a length of the complementary section of a maximum of 49 base pairs a efficient inhibition of the expression of the target gene can be achieve. Corresponding oligoribonucleotides can be made available at a low production cost. **(Note: This paragraph has a line by its side, perhaps to draw attention to it)**.

According to further requirements of the invention the use of double stranded oligoribonucleotides is necessary for the production of a drug for the inhibition of the expression of a given gene, where one strand of the dsRNA is at least partially complementary to this gene. Surprisingly such a dsRNA is suitable for the production of a drug for the inhibition of the expression of a given gene. When using dsRNA the inhibition takes place with a concentration that is at least one order of magnitude smaller than the concentration needed of a single stranded oligoribonucleotide. The procedure

associated with the invention therefore makes the production of especially effective drugs possible.

According to further requirement of the invention, the use of a vector to code double-stranded oligoribonucleotides (dsRNA) for the production of a medicine for the inhibition of the expression a given gene is prescribed, where a strand of the dsRNA is at least partially complementary to the gene. The use of a vector makes a very effective gene therapy possible.

When considering the advantageous designs of the medicine and its use reference will made to the preceding characteristics.

Embodiment

By means of conventional methods one of the only sequence protocol apparent RNA single strands was enzymatically synthesized.

In addition the RNA single strand complementary to it was synthesized. Subsequently the single strand and the single strand complementary to it was combined into dsRNA. The dsRNA produced contains a particular DNA sequence onder the control of the "immediate early gene" promoter of the cytomegalievirus.

Experimental Protocol

A plasmid vector was constructed, with which the necessary dsRNA could be produced, For its construction oligodesoxyribonucleotides were used as primers for a polymerase chain reaction (PCR), of which one sequence contained a EcoRI interchange and the T7-RNA-polymerase-promoter (5'- GGA ATT CTA ATA CGA CTC ACT ATA GGG CGA TCA GAT CTC TAG AAG – 3'), and the other contained a BamHT-interchange and the SP6-RNA-Polymerase-promoter (5' – GGG ATC CAT TTA GGT GAC ACT ATA GAA TAC CCA TGA TCG CGT AGT CGA TA-3'). Furthermore this primers contain identical or complementary areas on their 3'-ends for the "positive control DNA' of the HeLaScribe Nuclear Extract *in vitro* transcription kit of the firm Promega, that can be acquired by purchase, that also serves as a template for the PCR. The length of the DNA fragment amplified in this way was 400 base pairs, where 340 base pairs qualified as "positive control DNA". After PCR the product was cut with EcoRI and BamHI. The vector pUC18 served as a cloning vector for the PCR product obtained. A transformation of E. coli XL1-blue took place. Plasmid DNA of a chosen clone, whose sequence was reviewed using partial sequencing, was linearized using EcoRI and BamHI and was used as a stencil for a *in vitro* transcription with SP6- and T7-RNA-polmerase (riboprobe in vitro Transcription Systems, Firm Promega).

For hybridizing the following occured: 500 µL of single strands RNA kept in ethanol and was precipitated through centrifugation, the dried pellet was taken up in 30 µL PIPES-buffer (ph 6.4) in the presence of 80% Formamid, 400 mM NaCl and 1mM EDTA, 15 µL of the complementary single strands was put together and heated for 10 minutes at 85 degrees Celsius. Next the rudiments were incubated at 50 degrees Celsius overnight and

was then cooled to room temperature. To maintain pure dsRNA, the single strand RNAs were broken down by single strand specific RNase. For this 300µL Tris, ph 7.4, 300mM NaCl and 5mM EDTA 1,2 µl RNaseA (10 mg/ml) and 2 µL RNaseT1 (290 µL/ml was added to all rudiments. The rudiments were incubated for 1.5 hours at 30 degrees Celsius. Afterwards the RNasen were denatured by adding 5 µL Proteinase K (20 mg/ml) and 10 µL 20% SDS and incubated for 30 min at 37 degrees Celsius. The dsRNA was purified using phenol extraction and precipitated with ethanol. The tried pellet was taken up in 15 uL TE-buffer, pH 6.5.

Test System with human cell nucleus extract

While using the HeLaScribe Nuclear Extract in vitro transcription kit of the firm Promega the transcription efficiency of the DNA fragment mentioned above ("positive control DNA") was determined in the presence of both single stranded oligoribonucleotides as well as the dsRNA. This took place by means of the radioactivity of the GTP used as a substrate incorporated into the "run off" transcripts. The separation of the free GTP of the transcript created was made by gel electrophoresis. The analysis of the gel occurred with the help of a radioactivity detector (instant imager).

Result and Conclusion

A clear decrease in the amount on the transcript in the presence on dsRNA was seen in comparison to the control rudiment with RNA as well as to the rudiments with single stranded RNA. The effectiveness of the dsRNA could be reached even with the addition of small amounts (approximately 1 µL) The inhibiting effect of single stranded anti sense RNA could not be proven in this system as the inhibition takes place on the level of the translation. The transcription was examined here. The hereby first time reduction in a human cell of the transcript amount of a gene in the presence of dsRNA clearly shows an inhibition of the expression of the corresponding gene. This effect can be lead back to a new mechanism contingent on dsRNA.

Cover sheet

Federal Republic of Germany

Certificate

Dr. Roland Kreutzer in Weidenberg, Germany and Dr. Stefan Limmer in Bayreuth, Germany handed in a patent application with the title:

"Treatment and medicine for the inhibition of the expression of a of a given gene"

on November 24 1999 to the German Patent and Trademark office and declared that they have taken into consideration the inner priority of the application in the Federal Republic of Germany from January 30 1999, file 199 03 713.2.

The attached piece is a correct and precise rendition of the previous document of this patent application.

The application has provisionally received the designation C 12 N and A 61 K of the international patent classification.

Munich, the May 11 2000
German Patent and Trademark office
The President
By order of

(signature)

(printed name partially missing)

File: 199 56 568.6